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Significance of ABC Transporters in Fungicide Sensitivity and Resistance*

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Abstract: ATP-binding cassette (ABC) transporters are members of a protein superfamily which can be responsible for efflux of drugs from cells of target organisms. In this way, the transporters may provide a mechanism of protection against cytotoxic drugs. In laboratory-generated mutants of fungi, over-production of ABC transporters can cause multi-drug resistance to azoles and other non-related toxicants. The impact of this mechanism of resistance in field populations with decreased sensitivity to azoles remains to be established. Inhibitors of ABC transporter activity may synergize activity of azoles to populations of both sensitive and azole-resistant pathogens. The natural function of ABC transporters in plant pathogenic fungi may relate to transport of plant-defence compounds or fungal pathogenicity factors. Therefore, inhibitors of ABC transporter activity may act as disease control agents with an indirect mode of action.

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1 INTRODUCTION

ATP-binding cassette (ABC) transporters comprise the largest protein family known to date. More than 150 different ABC proteins in a wide variety of organisms have been identified, operating from bacteria to man.[1] It is generally accepted that these proteins can play a role in wild-type sensitivity of organisms to cytotoxic drugs. However, ABC transporters became known especially for their role in multi-drug resistance (MDR) of mammalian tumour cells.[2] MDR is the simultaneous resistance of cells to multiple, chemically unrelated drugs, observed after selection with a single cytotoxic drug. MDR is conferred by over-production of plasma membrane ABC transporters, actively secreting drugs. The over-production results in a decrease of intracellular concentrations of drugs so that these compounds cannot saturate their target sites and cells become resistant to cytotoxic drugs.

At least two types of ABC protein can confer MDR: a 170 kDa protein, often described as P-glycoprotein or MDR protein and a 190 kDa multi-drug resistance-associated protein (MRP).[3,4] Similar proteins have now been described in various mammalian species, and also in invertebrates such as *Caenorhabditis elegans* Maupas and *Drosophila melanogaster* Meig., and in parasitic protozoa such as *Leishmania tarentolae* and *Plasmodium falciparum* (Welch).[2] Data on the presence of MDR-like transporters in micro-organisms is rather limited and, with respect to fungi, particularly reported for yeasts such as *Candida albicans* (Robin) Berkhout, *Saccharomyces cerevisiae* Meyer ex Hansen and *Schizosaccharomyces pombe* Lindner.[5–7] Various research groups also focus their research on MDR-like transporters in filamentous fungi, especially mammalian

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pathogens. The first paper on the role of ABC transporters in MDR of fungi concerns *Aspergillus nidulans* (Eidam) Winter.[8] The first bacterial ABC transporter involved in MDR was described for *Lactococcus lactis*.[9]

This paper gives a short account of MDR in fungi and a description of the different models of ABC transporters which can be involved, especially those reported for yeasts. The recently reported ABC transporters of *A. nidulans* are also described. This fungus is regarded as a good model organism with which to study the significance of ABC transporters in fungicide sensitivity and resistance, since genetically defined mutants resistant to azole fungicides have been characterized. Research with *A. nidulans* paves the way to performing similar investigations with plant pathogens such as *Botrytis cinerea*. Pers. ex Fr. This organism will also be used to test the hypothesis that ABC transporters can play an important role in pathogenesis, and considerations supporting this hypothesis are also described in this paper.

2 MODEL OF THE ABC TRANSPORTER INVOLVED IN MDR

The model of the majority of ABC transporters involved in MDR is a plasma membrane-bound protein of *c.*1280 amino acids with 12 predicted trans-membrane domains in two homologous halves, each containing six predicted trans-membrane domains (TMD_6), preceded or followed by a conserved cytosolic nucleotide-binding fold (NBF or ATP-binding domain). Most ABC transporters of mammals and yeasts have a $[TMD_6\text{-}NBF]_2$ and a $[NBF\text{-}TMD_6]_2$ configuration, respectively.[1,2,10,11] Half-sized ABC proteins with a single TMD_6-NBF or NBF-TMD_6 configuration also occur.[9,11] The half-sized proteins are assumed to function after dimerization. ABC drug transporters can detect and expel a wide variety of drugs as they enter the plasma membrane and are, therefore, also described as 'hydrophobic membrane cleaners'.[2] Common characteristics of drugs transported by ABC drug transporters may be their hydrophobic and amphipathic properties. The energy required for drug transport is derived directly from ATP hydrolysis.

MDR cell lines possess relatively large amounts of ABC transporters in their cell membranes which, in turn, results in increased drug efflux. The overproduction of ABC proteins in MDR cell lines can be the result of increased transcription of genes encoding ABC genes, which can be the result of gene amplification (mammals) or be due to point mutations in transcription regulatory loci (*S. cerevisiae*).[2,5]

3 ABC TRANSPORTERS FROM YEAST

In *S. cerevisiae* no less than 16 ABC genes have been identified, while in-silicio analysis suggests that many more genes are present.[11] The first ABC transporter found in yeast is the *STE6* gene product, a $[TMD_6\text{-}NBF]_2$ protein not involved in MDR but responsible for the secretion of the mating type pheromone **a**.[12] Well-known multi-drug transporters of yeast are $[NBF\text{-}TMD_6]_2$ proteins encoded by *PDR5* (=*STS1* and *YDR1*) and *SNQ2*.[13,14] Disruption of either gene is not lethal but correlated to hypersensitivity to various compounds such as the antibiotic cycloheximide, the azole fungicide miconazole, and the mutagen 4-nitroquinoline *N*-oxide. *PRD5* and *SNQ2* gene transcripts are overexpressed in MDR mutants which carry point mutations in *PDR1*, encoding a transcription regulator of *ABC*-encoding genes.[13,15]

Multi-drug resistance-associated proteins (MRP) form a subfamily of ABC transporters. The first *MRP* identified in yeasts was *YCF1* from *S. cerevisae*, which encodes a TMD_4-$[TMD_6\text{-}NBF]_2$ protein.[16] A major difference from other ABC transporters is the presence of a TMD_4 at the *N*-terminal end. The transporter has been identified as a vacuolar glutathione *S*-conjugate pump, involved in protection against cadmium, drugs and other stress-metabolites.[17]

Drug transporters in *C. albicans* are BEN^r, a major facilitator drug transporter of benomyl and methotrexate, and CDR1, an ABC transporter of azole antimycotics and many other antifungal agents.[6,18,19] Recent research shows that most isolates of *C. albicans* from AIDS patients with resistance to azoles fail to accumulate these antimycotics.[20] The phenomenon is linked to an increase in the amounts of transcripts of either BEN^r or *CDR1*.

Finally, a number of *ABC* genes have been identified in *S. pombe*. One of them is $bfr1^+$, which confers resistance to brefeldin A and cycloheximide.[7]

4 MULTI-DRUG RESISTANCE IN FILAMENTOUS FUNGI

Several lines of evidence indicate that resistance to azole fungicides in laboratory-generated mutants of fungi are cases of MDR.[21] Phenotypic evidence is based on the observation that resistance to azoles can have pleiotropic effects. This has been demonstrated especially for *A. nidulans*, in which mutations for resistance to azoles in single loci may confer resistance or hypersensitivity to unrelated toxicants such as cycloheximide. Genetic evidence indicates that MDR in *A. nidulans* has a polygenic basis, resembling the situation described for *S. cerevisiae*.[10,22] Biochemical evidence for active secretion mechanisms of azoles in *A. nidulans* is also abundant, especially in azole-resistant isolates. It was shown unequivocally that resistance to azoles is correlated with decreased accumulation of azoles in mycelium, caused by an energy-dependent efflux activity.[23–27] In retrospect, this is a most striking resemblance to MDR

of mammalian tumour cells. This resemblance, and the rapidly increasing knowledge of molecular genetics of genes encoding ABC transporters and their regulatory genes, prompted us to continue our studies on fungal resistance to azoles, following a molecular approach.

5 GENES ENCODING ABC TRANSPORTERS FROM *ASPERGILLUS NIDULANS*

Genes encoding ABC transporters from *A. nidulans* were isolated by heterologous hybridization of a genomic phage library with a *PDR5*-derived probe from *S. cerevisiae*. The screening led to the identification of two single-copy genes, coded *atrA* and *atrB*.[8] The encoded proteins have the $[NBF\text{-}TMD_6]_2$ configuration and show a high degree of homology with PDR5, SNQ2 and CDR1. They all share the highly conserved Walker A and B motif and the ATP signature in the two NBFs. A minor difference from the yeast genes is the presence of four introns in *atrA* and three in *atrB*. Percentages of amino acid similarity and identity between AtrA and AtrB are 58 and 38, respectively. The encoded proteins can be regarded as new members of the ABC superfamily of transporters. Their relevance for fungicide transport was demonstrated by the observation that short treatments with the azoles imazalil and fenarimol enhanced transcription of the genes, coinciding with the transient induction of azole efflux in wild-type isolates reported earlier.[23,24] *ImaA* or *imaB* genes in isogenic strains of *A. nidulans* which cause resistance to azoles, strongly influence the basal level of expression of the *atr* genes and the capacity of azoles to enhance transcription.[8] Further functional analysis demonstrated that cDNA of *atrB* can also complement drug hypersensitivity in *S. cerevisiae*, determined by PDR5 deficiency.[8,22] These data indicate that ABC transporters of *A. nidulans* can play a significant role in fungicide sensitivity and resistance. This role can be studied further by gene disruption and phenotypic characterization of the knock-out mutants.

6 THE PHYSIOLOGICAL FUNCTION OF ABC TRANSPORTERS

Although ABC transporters can also be involved in transport of non-toxic substrates, the major part of these proteins function as drug transporters. However, synthetic drugs cannot be their natural physiological substrates. It is, therefore, believed that a natural physiological function of drug transporters is to protect organisms against toxic compounds occurring in nature, for example, endogenously produced toxins such as antibiotics.[28] Protection can also concern exogenous toxins to which organisms are exposed. For instance, ABC drug transporters of mammals may play a role in protection against toxic products present in their diet, and in the soil nematode *C. elegans* against toxic compounds made by plants and microbes in the rhizospere.[29–31] A similar reasoning may apply for many other organisms, including filamentous fungi.[21] During pathogenesis, plant pathogenic fungi are exposed to fungitoxic plant defence products (phytoncides and phytoalexins). Successful plant pathogens do cope with these compounds. We propose that a natural function of ABC transporters of plant pathogens is protection against these plant defence products. Fungal ABC transporters may accept these products as substrates, thereby limiting their accumulation in mycelium and avoiding their toxic effects. Circumstantial evidence supporting this hypothesis is the observations that mutants of *A. nidulans* carrying *imaB* genes for resistance to azoles are less sensitive to pisatin.[8] Simultaneous resistance to azoles and isoflavonoid phytoalexins has previously been observed in *Cladosporium cucumerinum* Ell. & Arth.[32] Pisatin also rapidly induces higher transcript levels of *atrB* in *A. nidulans*.[8] The hypothesis also corroborates the finding that *Nectria haematococca* Berk. & Broome possesses an inducible and energy-dependent mechanism that secretes pisatin from mycelium.[33] Another, more speculative function of ABC transporters in pathogenesis could be the secretion of particular pathogenicity factors (toxins, peptides) from plant pathogens. The hypotheses are currently tested using *B. cinerea* as a test organism.[34] This pathogen was selected because of its broad host range, implying the potency to cope with a wide variety of plant defence products. The pathogen also produces various pathogenicity factors. One of the strategies to test the hypotheses described is by determining the virulence of mutants with a disruption in genes encoding ABC transporters.

7 DISCUSSION

We hypothesise that ABC transporters in fungi can play a significant role in fungicide sensitivity. A low sensitivity may be caused by a high level of ABC transporter activity, resulting in low accumulation levels of fungicides in mycelium and natural insensitivity of a fungus despite the presence of a sensitive target site. In contrast, a low level of ABC transporter activity may facilitate accumulation and hence may enhance sensitivity. Evolution of resistance to agricultural fungicides (e.g. azoles) can be caused by mutations causing overexpression of *ABC* genes, resulting in an overproduction of ABC transporters and exclusion of azoles from fungal cells. This mechanism operates in laboratory-generated mutants of various fungi, including plant pathogens, but remains to be established for field-resistant mutants.[23–27] Increased ABC transporter activity has been reported as a mechanism of resistance

to azole antimycotics in clinical isolates of *C. albicans* from AIDS patients.[19,20] This observation suggests that the mechanism may also be relevant in azole-resistant field isolates of plant pathogens. We also propose that inhibitors of activity of ABC transporters can act as synergists of azoles in fungi with a high level of ABC transport activity. These inhibitors may synergize efficacy of azoles in azole-resistant populations, provided that the resistance mechanism operating is based on increased ABC transport activity of azoles.[21,35]

Generally, disruption of *ABC* genes is not lethal.[15,29–31] This implies that specific inhibitors of ABC transporter activity are not toxic and, more specifically, that inhibitors of ABC transport activity in fungi have no in-vitro toxicity. However, such inhibitors may have a disease control activity if fungal ABC transporters indeed have a natural physiological function in accumulation of plant defence compounds in fungal cells or in secretion of pathogenicity factors. In the first case, accumulation of plant defence factors in mycelium would not be reduced any more by active efflux. As a result, natural defence reactions may become more effective and lead to host resistance. In the second case, inhibition of ABC transporters would result in decreased secretion of pathogenicity factors as a result of which pathogens may lose their virulence. For these reasons, we also propose that specific inhibitors of ABC transporter activity in fungi can act as leads in the discovery of disease-control agents with an indirect mode of action.[21,35] The risk of evolution of resistance to such compounds in plant pathogens might be much less than to fungicides with a direct mode of action.

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